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COMMUNICATION

A thermostable enzyme as an experimental platform to study properties of less stable homologues

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The structural and functional characterization of proteins is frequently hampered by lack of stability or by insufficient assembly of oligomeric proteins in over-expression systems. Using F_1-ATPase as a case study, we tackled this problem by introducing function-determining domains from a difficult-to-handle variety of an enzyme into a stable homologue.
Keywords: chimeric enzyme/protein assembly/protein engineering/protein stability

Introduction

Proteins from thermophilic organisms are widely used in basic and applied research, e.g. for structural studies, investigation of structure–function relationships or for processes involving long-term usage or storage (for reviews, see Yano and Poulos, 2003; Haki and Rakshit, 2003). Owing to their superior stability, they are frequently used as model systems for less stable homologous proteins from other organisms. However, in many cases such related enzymes display different or additional catalytic or regulative properties, questioning this model system approach. If a high-resolution structure is available for an unstable enzyme, computer-aided protein design techniques can be applied to predict stabilizing mutations. Here we describe an alternative approach that may be especially useful if no high-resolution structures are reported and also for large, oligomeric proteins, for which it is difficult to predict which factors determine stability. Two varieties of F_1-ATPase serve as a case study for utilizing a thermostable protein as an experimental scaffold to which domains of a less stable homologue have been added. The resulting enzymes, while retaining their superior stability, allow for functional investigation of the introduced properties.

Problem

The F_1-ATPase part of F_0F_1 ATP synthase is a large oligomeric protein complex with the main subunit composition $\alpha_3\beta_3\gamma$ and a molecular mass of about 400 kDa (Figure 1A) (for reviews see Boyer, 2000; Yoshida *et al*., 2001). Constituting the key enzyme for energy conversion in living cells, F_0F_1 is regarded a paradigm for mechanical energy conversion in biology. Its F_1-ATPase part utilizes ATP hydrolysis to drive subunit rotation and might even take a central role as an energy delivery device in future nanotechnology. DNA sequencing revealed a high degree of amino acid sequence similarity between F_1-ATPases from bacteria, plants and mitochondria (Saraste *et al*., 1981; Cozens and Walker, 1987; Miki *et al*., 1988). Nevertheless, F_1-ATPases from different organisms display varying properties concerning stability, assembly and regulation: whereas assembly of F_1-ATPase from plant chloroplast (CF_1, Figure 1A, left) remains an obstacle, its homologue from the thermophilic *Bacillus* PS3 (TF_1, Figure 1A, right) readily assembles from its subunits in a standard *Escherichia coli* over-expression system (Matsui and Yoshida, 1995). CF_1 is unstable at room temperature and even dissociates into its subunits at temperatures below 10°C (Hightower and McCarty, 1996). In contrast, TF_1 is stable at temperatures from below 4°C up to 75°C and therefore suitable for long-term usage and storage (Yoshida *et al*., 1975). On the other hand, CF_1 features several important properties, which have remained largely elusive owing to difficulties in handling, genetic manipulation and usage in long-term experiments. In contrast to F_1-ATPases from other sources CF_1 contains an additional region comprised of 35–40 amino acid residues and responsible for redox-dependent regulation (Figure 1A, left) (Nalin and McCarty, 1984; Miki *et al*., 1988). Oxidation and reduction of an internal disulfide bond located in this regulatory region modulate enzyme activity in response to the cell's reducing potential and plays a key role in the switching of plants between day and night metabolism (Nalin and McCarty, 1984). A second unique feature is the sensitivity of some varieties of CF_1 to tentoxin, a cyclic tetrapeptide produced by phytopathogenic fungi: whereas low concentrations of this inhibitor block enzyme activity, high concentrations surprisingly re-activate the enzyme. In addition to our fundamental interest in understanding structural and functional aspects of such inhibitory mechanisms, particularly in the light of prospective future usage of this enzyme in nanotechnology, controllability of activity constitutes an issue of high priority.

Experimental strategy

In order to allow the elucidation of these two unique properties of CF_1, we utilized the highly stable TF_1 as a platform to incorporate the respective structural elements. In a first approach, we attempted to transplant the redox-regulation feature (Figure 1B). Since no high-resolution structure was available for the respective parts of either enzyme, we based our strategy on an amino acid sequence alignment (Figure 1B). A piece of the TF_1 γ subunit (from Asn94 to Glu205) was replaced with the corresponding part from CF_1 γ (from Met95 to Glu243) containing the regulatory region (Bald *et al*., 2000). To prevent structural incompatibility, the incorporated fragment was flanked by stretches of

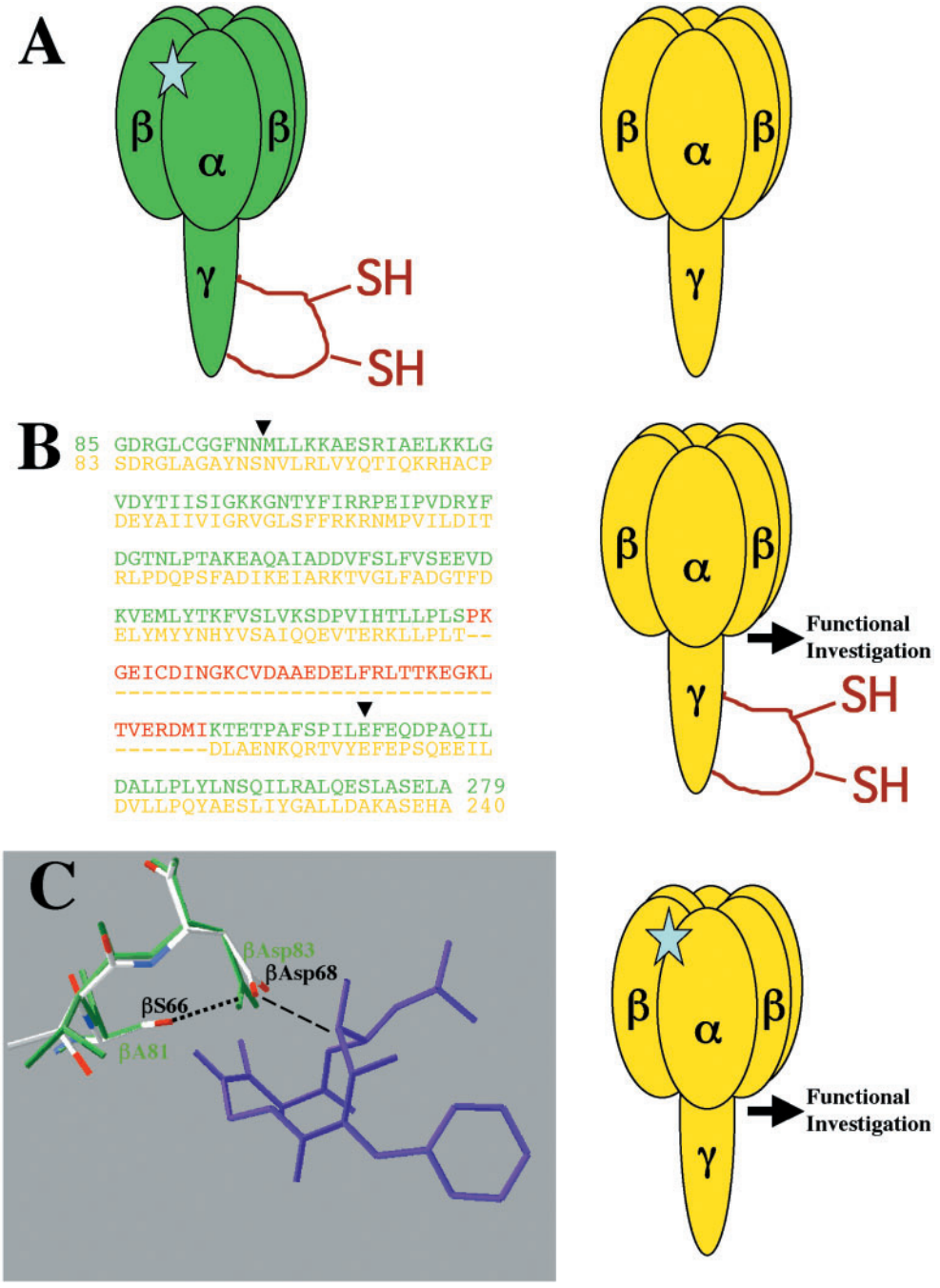

Fig. 1. Incorporation of properties from chloroplast F_1-ATPase into thermostable F_1-ATPase. (**A**) Comparison of F_1-ATPase from spinach chloroplast (CF_1, green) and thermostable F_1-ATPase (TF_1, yellow). CF_1 contains as special features an additional regulatory region (red) and sensitivity to tentoxin (star), lacking in TF_1. TF_1 is used here as a platform to which features of CF_1 can be attached and subsequently investigated. (**B**) Based on an amino acid sequence comparison the regulatory region was incorporated. The sequence of CF_1 is shown in green, its regulatory region in red and TF_1 is depicted in yellow. A fragment indicated by the arrows was transferred from CF_1 to TF_1. (**C**) Based on a structure overlay of the tentoxin-binding region (left, green, CF_1; purple, tentoxin; CPK, TF_1), tentoxin sensitivity (star) was incorporated into TF_1 by the point mutation βSer66Ala (right). In CF_1, βAsp83 forms a hydrogen bond with tentoxin (left, dashed line). In TF_1, the hydrogen bond between βAsp66 (corresponding to βAsp83 in CF_1) may be weakened by a competing hydrogen bond (dotted line) with βSer66 (corresponding to βAla81 in CF_1).

comparatively high amino acid sequence identity. The replacement was carried out at DNA level, substituting part of the gene coding for the γ subunit of TF_1 by the corresponding DNA fragment coding for part of CF_1 γ. The newly constructed gene was over-expressed in *E.coli* (Bald *et al*., 2000) using a system established for TF_1 (Matsui and Yoshida, 1995). The resulting protein (Figure 1B) was sensitive to redox regulation in a fashion comparable to CF_1.

In a second approach, we rendered TF_1 sensitive to tentoxin (Figure 1C). For the respective regions of both CF_1 and TF_1, high-resolution structures are available (Shirakihara *et al*., 1997; Groth and Pohl, 2001). A 3-D structure of CF_1 complexed with one molecule of tentoxin resolved at 3.4 Å provided insight into the location of the tentoxin binding site and suggested an important role of several amino acid residues in its vicinity for efficient binding (Groth, 2002). A structural

alignment with the 3-D structure of TF_1 suggested that coordination of tentoxin by a critical aspartate (βAsp83 in CF_1, βAsp68 in TF_1) might be disturbed by an adjacent serine residue in TF_1 (Figure 1C). Replacement of this residue by alanine (as found in CF_1) strongly increased TF_1 sensitivity for tentoxin with a degree of inhibition comparable to CF_1 (Groth *et al*., 2002).

Incorporation of both redox sensitivity (Figure 1B) and tentoxin sensitivity (Figure 1C) into TF_1 were successfully carried out without impeding assembly of the enzyme from its subunits (Bald *et al*., 2000; Groth *et al*., 2002). Furthermore, the chimeric enzymes were as thermo- and cold stable as TF_1. Transfer of features from CF_1 to TF_1, even when involving a large domain (Figure 1B) thus did not diminish its usage in long-term experiments at room temperature. These findings also indicate that the regulatory region and the tentoxin-binding site special to CF_1 are not responsible for the enzyme's lack of assembly or stability. Construction and characterization of such chimeric proteins have been described earlier for the investigation of, e.g., heat stability of RNase (Schulga *et al*., 1998) and isopropyl malate dehydrogenase (Numata *et al*., 2001), as well as assembly of F_1-ATPase (Burkovski *et al*., 1994; Hisabori *et al*., 1997), and constitutes a useful method to pinpoint regions or amino acid residues conferring stability or inhibitor sensitivity to a protein. We have now extended the usage of such chimeras by investigating the functional implications of the introduced features, which was not possible with either original protein.

Investigation of incorporated features

F_1-ATPase works as a rotary motor enzyme. Rotation of the γ subunit relative to the $\alpha_3\beta_3$ part has been demonstrated after tight immobilization of the enzyme and attachment of large probes to the γ subunit of TF_1 (Yasuda *et al*., 2001). After transplantation of CF_1 features to TF_1, these advanced methods have been used to study redox regulation and the impact of tentoxin binding on single enzyme molecules. The characterization of individual molecules under reducing (high activity) and oxidizing (low activity) conditions revealed that the observed incomplete inactivation of CF_1 is attributable to oscillation of the oxidized enzyme between a fully active and a fully inactive state (Bald *et al*., 2001). Investigation of single tentoxin-sensitive F_1-ATPase molecules in the presence of varying tentoxin concentrations revealed that low concentrations (binding of one tentoxin molecule per enzyme molecule) completely inhibited the enzyme. Re-activation achieved with high tentoxin concentrations (attributed to 2–3 molecules of tentoxin bound per enzyme molecule) restored working of the enzyme, but in a different functional mode compared with the uninhibited enzyme (Pavlova *et al*., 2004).

Outlook

Enzymes from bacteria or lower organisms are frequently used as model systems to investigate homologous enzymes from other sources. In the fairly frequent case that any given model enzyme does not have the desired property, the strategy described here should prove highly useful. We are currently working towards the generation of chimeras with much larger substituted areas, employing complementation and artificial evolution assays to secure compatibility. This will allow us to extend our focus and to study features with more diffuse structural localization. For example, introduction of large stretches unique to mitochondrial F_1-ATPase into the TF_1 scaffold may turn out to facilitate investigation of the recently reported interaction of this enzyme with the blood-pressure regulator angiostatin (Moser *et al*., 2001) or the cholesterol effector apo-lipoprotein A-I (Martinez *et al*., 2003).

In addition to this more biochemical focus, incorporation of regulative features into a stable protein scaffold has been used to influence the movement of F_1-ATPase (Bald *et al*., 2001; Pavlova *et al*., 2004; Ueoka-Nakanishi *et al*., 2004) This may well form a basis for future application of motor proteins in nano-biotechnology, e.g. by allowing control of the motion of recently reported biological/inorganic hybrid nano-devices (Soong *et al*., 2000; Liu *et al*., 2002).

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